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Inosine Analogs as Chemotherapeutic Agents for African Trypanosomes: Metabolism in Trypanosomes and Efficacy in Tissue Culture

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Certain purine analogs, the pyrazolopyrimidines, are effective chemotherapeutic agents against *Leishmania* spp. and *Trypanosoma cruzi* both in vitro and in some clinical models. Heretofore they have not been effective against the African trypanosomes; this suggested that these organisms were not comparable to the other pathogens with respect to their purine metabolism. We have studied the efficacy and metabolism of the pyrazolopyrimidine bases allopurinol and thiopurinol, their respective ribonucleosides, and the C-nucleosides formycin B and 9-deazainosine in *Trypanosoma brucei* subsp. *gambiense* and *Trypanosoma brucei* subsp. *rhodesiense*. The efficacy of these compounds was dependent on the purine content of the culture medium. The C-nucleosides were the most effective, with 90% effective doses for formycin B and 9-deazainosine of 0.01 and 2 μg/ml, respectively. Metabolism was the same in both the bloodstream and culture forms and identical to that reported for *Leishmania* spp. and *T. cruzi*. Both agents were phosphorylated to the ribonucleotide and then aminated to produce adenine nucleotide analogs. Growth inhibition studies were performed with three inosine analogs (allopurinol riboside, formycin B, and 9-deazainosine) on trypomastigotes grown in bone marrow tissue culture. Both C-nucleosides eradicated the infection at a concentration of 0.25 μg/ml. Unlike formycin B, 9-deazainosine is not known to be aminated by mammalian cells and appears to be relatively nontoxic in three different mammalian tissue culture systems. This nucleoside was very active against all pathogenic leishmaniae and trypanosomes investigated and is worthy of further study.

Certain hypoxanthine and inosine analogs (Fig. 1) are biologically active against the culture forms of *Leishmania* spp. and *Trypanosoma cruzi* (11). Procyclic forms of the African trypanosomes are only slightly susceptible to the hypoxanthine analog allopurinol, but convert it to the same unique metabolic products as the above organisms (3). Since purines reverse the antibiological activity of these analogs (11), we reexamined the biological effects of allopurinol and other purine analogs on the procyclic forms of *Trypanosoma brucei* subsp. *gambiense* and *Trypanosoma brucei* subsp. *rhodesiense* in a medium with a lower purine content than that used previously (9). In addition, we investigated the metabolism of a selected inosine analog in both the procyclic and bloodstream forms of these parasites. Agents that were active against the procyclic forms and were similarly metabolized in both the procyclic and bloodstream forms were tested for efficacy against trypomastigotes grown in bone marrow tissue culture.

MATERIALS AND METHODS

Culture technique. *T. brucei* subsp. *gambiense* procyclic (strain TH114) and bloodstream (strain TTrT-8) forms and *T. brucei* subsp. *rhodesiense* procyclic (strain STIB364) and bloodstream (Liverpool strain) forms were studied. Growth, harvesting, and incubation were performed as previously described (8, 9). Medium (PDM-79) with a low or defined purine content was prepared by modifying the medium of Brun et al. (SDM-79) (7) as previously described (8, 9). This reduced the purine content to levels comparable to those of the media used in our drug efficacy tests with leishmaniae (2) and *T. cruzi* (4).

The growth inhibition studies were performed as previously described (3). The 90% effective dose (ED_{90}) (the drug concentration that caused a 90% reduction in growth compared with untreated control cultures) was determined from plots of cell growth versus drug concentration.

Investigations of the efficacy of certain compounds on the growth of the bloodstream forms of *T. brucei* subsp. *gambiense* [Wellcome TS (TXTaT-1) strain] were performed with bone marrow cultures prepared from BALB/c Cr mice by the method of Balber (1). For these tests, 96-well round-bottom tissue culture plates with confluent monolayers of marrow cells were inoculated with 10^3 parasites (maintained in bone marrow culture), drug was added at the desired concentration, and the plates were incubated at 35°C in a humidified incubator with a 5% CO_2 atmosphere. Growth was scored on day 5 by counting the number of parasites per well by inverted-phase microscopy. Control culture cell density was normally 10^6 cells per well at this time. Results for each drug concentration represent the average of five replicate experiments.

Drug metabolism. Endogenous nucleotides and purine analog metabolite pools were extracted, processed, and analyzed by ion-exchange high-pressure liquid chromatography as previously described (3, 8, 9). The identity of the metabolic products of the analogs was confirmed by comparison of their retention times and their absorbance ratios at 254 and 292 nm with authentic standards.

Materials. Labeled and unlabeled allopurinol (4-hydroxypyrazolo[3,4-*d*]pyrimidine [HPP]), allopurinol riboside (4-hydroxypyrazolo[3,4-*d*]pyrimidine ribonucleoside

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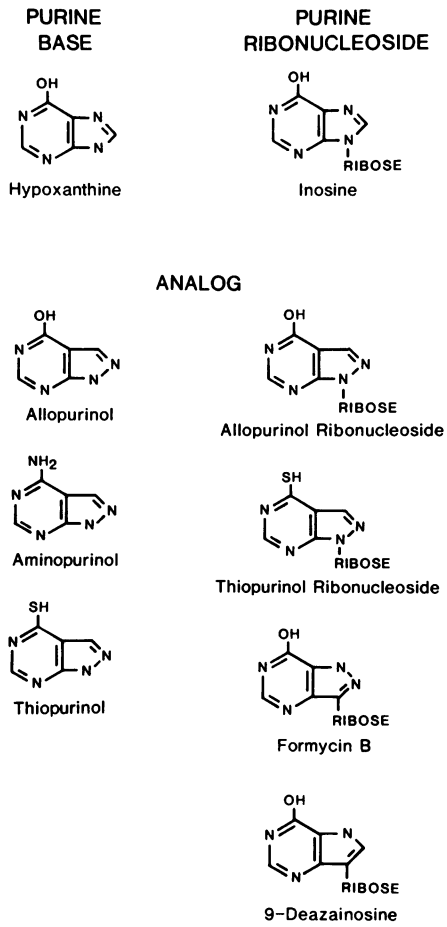

FIG. 1. Structures of certain purines and purine analogs.

[HPPR]), thiopurinol (4-thiopyrazolo[3,4-*d*]pyrimidine [TPP]), and thiopurinol riboside (4-thiopyrazolo[3,4-*d*]pyrimidine ribonucleoside [TPPR]) were obtained from the Burroughs Wellcome Co., Research Triangle Park, N.C.; radiolabeled formycin B (7-hydroxypyrazolo[4,3-*d*]pyrimidine ribonucleoside [FORB]) was from Moravek Biochemicals, Brea, Calif.; 9-deazainosine (9-DINO) was provided by Robert Klein, Sloan-Kettering Institute for Cancer Research, New York, N.Y.; culture media supplies were from Kansas City Biologicals, Inc., Lenexa, Kans.; plastic cultureware was from Costar, Cambridge, Mass.; and biochemicals were from Sigma Chemical Co., St. Louis, Mo. All other chemicals were analytical grade or better.

RESULTS

Effects of purine analogs on growth of procyclic forms. Table 1 shows the ED_{90} determined for the pyrazolopyrimidines HPP, HPPR, TPP, and TPPR and the C-nucleosides FORB and 9-DINO. The organisms were cultivated in low-purine PDM-79 (≈1 μM total purine concentration derived from the fetal bovine serum component). Of the drugs tested against *T. brucei* subsp. *gambiense*, the two C-nucleosides were the most effective and the thio compounds were the least effective. Results obtained with *T. brucei* subsp. *rhodesiense* differed from those obtained with *T. brucei* subsp. *gambiense* in that HPP and its riboside were less effective on this organism, whereas TPP was more effective. The remaining drugs were about equally effective against both organisms. Previous studies with *T. cruzi* indicated that the thio compounds were relatively inactive since

TABLE 1. ED_{90} for various purine analogs tested against the procyclic forms of *T. brucei* subsp. *gambiense* and *T. brucei* subsp. *rhodesiense*

Compound	*T. brucei* subsp. *gambiense*		*T. brucei* subsp. *rhodesiense*	
	μg/ml	μM	μg/ml	μM
HPP	12	88	34	250
HPPR	24	90	39.3	146
TPP	200	1,315	70	461
TPPR	50	178	47.5	168
FORB	0.01	0.04	0.01	0.05
9-DINO	2	7	2	8

their effects were reversed by the small amounts of adenine present in the fetal bovine serum (11). We investigated this possibility in *T. brucei* subsp. *gambiense* since the ED_{90} for both TPP and TPPR was quite high. When TPP was tested against *T. brucei* subsp. *gambiense* grown in purine-free PDM-79 supplemented only with hypoxanthine, the ED_{90} was reduced from 200 to 18 μg/ml, a value comparable to that found for HPP. When adenine was used as the purine supplement instead of hypoxanthine, the ED_{90} again was 200 μg/ml. Control cultures started at a cell density of $\approx 10^5$ cells per ml and grown in purine-free PDM-79 plus either adenine or hypoxanthine took approximately 1.5 times as long to reach the stationary phase of growth (11 days instead of 7), but the final cell density (ca. 3×10^7 cells per ml) was the same as that in cultures grown in regular PDM-79.

Metabolism of purine analogs in procyclic forms. Berens et al. (3) reported that incubation of procyclic forms with HPP for 24 h resulted in the formation of HPPR monophosphate (HPPR-MP) as well as the mono-, di-, and triphosphates of aminopurinol riboside (4-aminopyrazolo[3,4-*d*]pyrimidine ribonucleoside [APPR; APPR-MP, APPR-DP, and APPR-TP]). Similar incubations were done with *T. brucei* subsp. *gambiense* procyclic forms and the pyrazolopyrimidines HPPR, TPP, and TPPR (Table 2). HPPR was converted to the same metabolic products as HPP; both TPP and TPPR were converted only to TPPR-MP. These results are exactly those found for similar incubations with *Leishmania donovani* (14).

Table 3 shows the results of a similar experiment done with the C-nucleosides FORB and 9-DINO. Both these inosine analogs are metabolized in the same way as HPPR in that they are converted to their respective nucleoside monophosphates and then aminated to their respective analogs of AMP, ADP, and ATP. Only the ATP analog of 9-deazaadenosine was detected since 9-DINO-MP and the mono-and diphosphate forms of 9-deazaadenosine were in distinguishable from normal cellular nucleotides. Radiolabeled 9-DINO was not available.

Metabolism of purine analogs by the bloodstream forms. The bloodstream forms of *T. brucei* subsp. *gambiense* and *T.*

TABLE 2. Metabolism of pyrazolopyrimidines by procyclic forms of *T. brucei* subsp. *gambiense*

Metabolic product	Concn of metabolic product (pmol/10^6 cells)[a] from:		
	[^{14}C]HPPR	[^{35}S]TPP	[^{35}S]TPPR
HPPR-MP	54.6	—	—
APPR-MP	1.7	—	—
APPR-DP	2.8	—	—
APPR-TP	2.6	—	—
TPPR-MP	—	32.1	28.7

[a] Approximately 10^9 cells were incubated in 100 ml of PDM-79 for 24 h with each radiolabeled drug (93 μM; specific activity 1 μCi/1.9 μmol). —, None detected.

TABLE 3. Metabolism of the C-nucleosides FORB and 9-DINO by procyclic forms of *T. brucei* subsp. *gambiense*[a]

Metabolic product	Concn of metabolic product (pmol/10^6 cells) from:	
	9-DINO	FORB
FORB-MP	—[b]	6.2
FORA-MP	—	64.5
FORA-DP	—	11.4
FORA-TP	—	25.4
9-Deaza-ATP	8.4	—

[a] Conditions were as described for Table 2, footnote *a*, except the drug concentration was 37 μM and it was not radiolabeled.
[b] —, None detected.

TABLE 5. Metabolism of the C-nucleosides FORB and 9-DINO by bloodstream forms of *T. brucei* subsp. *gambiense*[a]

Metabolic product	Concn of metabolic product (pmol/10^6 cells) from:	
	9-DINO	FORB
FORB-MP	—[b]	3.3
FORA-MP	—	7.1
FORA-DP	—	0.9
FORA-TP	—	0.1
9-Deaza-ATP	5.8	—

[a] Conditions were as described for Table 4, footnote *a*, except the drug concentration was 37 μM and it was not radiolabeled.
[b] —, None detected.

brucei subsp. *rhodesiense* metabolized the pyrazolopyrimidines HPP, HPPR, and TPPR (Table 4) and the C-nucleosides FORB and 9-DINO (Table 5) exactly as the procyclic forms did.

Efficacy of HPPR, FORB, and 9-DINO on bloodstream forms growing in tissue culture. FORB and 9-DINO were the most effective compounds against the procyclic forms, and they were metabolized identically in both procyclic and bloodstream forms. For these reasons they were chosen for in vivo testing against bloodstream forms grown in a bone marrow tissue culture system. HPPR was tested to see whether it might be more effective against the vertebrate forms than it was against the procyclic forms (Table 6). HPPR was relatively ineffective against these organisms at concentrations of 10 μg/ml or less. The two C-nucleosides were both quite active at very low concentrations, with FORB somewhat more efficacious than 9-DINO.

DISCUSSION

Pyrazolopyrimidines which are effective against leishmaniae and *T. cruzi* (11) have little or no effect on the growth of *T. brucei* subsp. *brucei* or the STIB386 strain of *T. brucei* subsp. *rhodesiense* at concentrations as high as 200 μg/ml (3; Berens and Brun, unpublished data). When the medium was modified to reduce its purine content from 10 to 1 μM, the ED_{90} of these agents was significantly reduced (Table 1). One exception was the persistently high concentration of TPP required to inhibit *T. brucei* subsp. *gambiense*. Since the metabolic product of TPP, the IMP analog TPPR-MP, inhibits the enzyme that converts IMP to AMP (adenylosuccinate synthetase, EC 6.3.4.4) in leishmaniae (9), it was thought that any adenine in the medium could bypass this block through direct conversion to AMP. This process does not happen in leishmaniae since they possess an adenine aminohydrolase which rapidly converts most adenine to hypoxanthine (13). This enzyme is not present in the African trypanosomes (8, 9). The tests for the effects of TPP on *T. brucei* subsp. *gambiense* were repeated with modified medium in which hypoxanthine was the only purine source. Under these conditions the ED_{90} was reduced from 200 to 18 μg/ml. These results suggest that the adenylosuccinate synthetase of this organism also is sensitive to TPPR-MP inhibition. *T. cruzi* shows a similar resistance to TPP unless forced to grow on hypoxanthine (11). In addition, it was found that *T. brucei* subsp. *rhodesiense* cultured in PDM-79 was less sensitive to HPP and HPPR and more sensitive to TPP than *T. brucei* subsp. *gambiense*. The reason for this difference is unclear, but it may reflect quantitative differences in either transport or enzymatic activities between the two organisms. We found similar differences among various *T. cruzi* strains with respect to HPP and HPPR sensitivity (4a). These differences are currently under investigation. Although reduction or modification of the purine content of the culture medium resulted in increased sensitivity of the African trypanosomes to these pyrazolopyrimidines, they still were not as sensitive to these compounds as are leishmaniae.

Two inosine analogs, FORB and 9-DINO, were investigated since other pathogenic hemoflagellates are very sensitive to them (11, 12). Both are C-nucleosides, the ribose moiety of which is bound to the five-membered ring by a carbon-carbon bond instead of the normal carbon-nitrogen bond (Fig. 1). This arrangement renders these compounds poorly reactive to the enzymes responsible for converting purine ribonucleosides to their bases (18). Both were very effective against the procyclic forms of these organisms (Table 1); FORB was about 200-fold more effective than 9-DINO.

The metabolism of these compounds in procyclic forms is shown in Tables 2 and 3. HPPR underwent the same sequence of conversions as that found for HPP (3). Both FORB and 9-DINO were also converted to their respective IMP analogs and then aminated to their respective AMP, ADP, and ATP analogs. The thio compounds, TPP and

TABLE 4. Metabolism of pyrazolopyrimidines by bloodstream forms

Metabolic product	Concn of metabolic product (pmol/10^6 cells)[a] from:					
	T. brucei subsp. *gambiense*			*T. brucei* subsp. *rhodesiense*		
	HPP	HPPR	TPPR	HPP	HPPR	TPPR
HPPR-MP	4.8	13.4	—[b]	9.1	5.4	—
APPR-MP	0.8	0.4	—	1.0	1.2	—
APPR-DP	0.4	0.8	—	1.0	0.6	—
APPR-TP	0.1	0.3	—	0.5	0.4	—
TPPR-MP	—	—	108.9	—	—	21.9

[a] Approximately 5×10^8 freshly isolated bloodstream forms were incubated for 3 h in 10 ml of PDM-79 containing 200 μM radiolabeled drug ([^{14}C]HPP and [^{14}C]HPPR, specific activity, 1 μCi/μmol; [^{35}S]TPPR, specific activity, 1 μCi/1.3 μmol).
[b] —, None detected.

TABLE 6. Effects of HPPR, FORB, and 9-DINO on the growth of *T. brucei* subsp. *gambiense* bloodstream forms in bone marrow culture

Compound	Concn (μg/ml) required to produce:		
	Cure[a]	ED_{50}[b]	No effect
HPPR	ND[c]	ND	10
FORB	0.06	0.02	0.01
9-DINO	0.25	0.06	0.03

[a] No trypanosomes detected in microwell after 5-day culture.
[b] Drug concentration that caused a 50% reduction in growth compared with untreated control cultures.
[c] ND, Not done.

TPPR, were converted only to TPPR-MP as occurs in leishmaniae (14). The results with TPP are consistent with the ability of exogenous adenine in the medium to reverse this inhibition. TPP and TPPR are converted only to the monophosphate, which presumably inhibits adenylosuccinate synthetase and the formation of AMP from IMP. The cell is not starved for AMP since it can be formed directly from exogenous adenine. The other pyrazolopyrimidines inhibit adenine-related reactions also, but unlike TPP and TPPR, they are converted to their respective adenine ribonucleotide analogs. (11).

Although the conversion of HPP, HPPR, and 9-DINO to their respective adenine nucleotide analogs appears to be unique to hemoflagellates (11), the conversion of FORB to the formycin A (FORA) analogs of adenine nucleotides is not. FORB is known to inhibit several enzymes of nucleic acid metabolism (15, 16). Mouse L cells can phosphorylate FORB, convert the nucleotide to all of the corresponding FORA nucleotides, and incorporate the ATP analog into the RNA (17). This raises the possibility that FORA could be generated from FORB in humans. Indeed, Berman et al. (6) have demonstrated that FORA nucleotides can be formed from FORB in human macrophages; the toxicity of a series of formycin analogs for *Leishmania tropica* was paralleled by their toxicity for these macrophages (5). Glazer and Lloyd (10) have shown that FORA can be incorporated into the DNA of human colon carcinoma cells.

Metabolic experiments with freshly isolated bloodstream forms gave results comparable to those for the procyclic forms (Tables 4 and 5). The one surprising finding was the large amount of TPPR-MP formed by *T. brucei* subsp. *gambiense*, the reason for which is unclear. These data suggest that metabolic studies with compounds of this nature done with the procyclic forms will apply directly to the pathogenic bloodstream forms as well. This supposition was supported by the tissue culture experiments.

The C-nucleosides are metabolized to toxic products by both procyclic and bloodstream forms and appear to have the greatest potential for chemotherapy. HPPR, despite its low toxicity, required high concentrations to be effective. Both of the C-nucleosides eliminated bloodstream trypanosomes from the tissue culture system at very low concentrations (Table 6). Although FORB was more active than 9-DINO, both killed all trypanosomes at 0.25 μg/ml or less. FORB is toxic to mammalian systems for the reasons given above and does not appear to be useful for chemotherapy. The toxicity of 9-DINO is unknown, although it has shown no toxicity for mouse L cells at a concentration of 1 mM (12) and is not converted to its ATP analog in these cells (S. W. LaFon, N. K. Cohn, and D. J. Nelson, Fed. Proc., **42**:2006, 1983). There is no visible evidence of toxicity for VA-13 (human diploid lung) or U937 (human histiocytic lymphoma) cells at 0.1 mM (25 μg/ml) (unpublished data). This inosine analog is worthy of further study.

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